The Effect of the Injection of Intracellular Constituents of Bacteria (Bacterial Endotoxins) on the Opsonising Action of the Serum of Healthy Rabbits.

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In a series of researches the late Dr. Allan Macfadyen studied the properties of the intracellular constituents of bacteria and other organisms obtained by mechanical trituration of the organisms in the presence of liquid air. He showed that the cell juices thus obtained are:—

(1) Toxic on injection into animals (*e.g.*, *B. typhosus*,* *Spirillum choleræ*,† *B. suisepticus*,‡ pneumococcus,§ and others).

(2) Capable of inducing the formation of anti-endotoxins on injection into animals (*e.g.*, *B. typhosus*,‖ *Spirillum choleræ*¶) which possess protective and curative properties *in vivo*, and bacteriolytic properties *in vitro*.

(3) Cause the development of agglutinins (*e.g.*, *B. typhosus*** and yeast††). It was thought that it might be of interest to investigate whether the intracellular bacterial constituents are capable of inducing alteration in the opsonising action of the serum of normal rabbits.

The organisms selected were the *B. typhosus*, the *B. tuberculosis*, and the *M. pyogenes*, var. *aureus* (*Staphylococcus pyogenes aureus*). The intracellular constituents of these organisms were obtained by the Macfadyen method,‡‡ viz., by growing the organism on surface agar in Roux bottles, scraping off the growth, suspending this in sterile water, centrifugalising and collecting the bacterial paste on the walls of the centrifuge. The bacterial paste is weighed so as to ascertain the amount, and then ground in the machine after freezing.

After grinding, the ground material is made up with distilled water or with 0·1-per-cent. sodium hydrate, so as to form a 10-per-cent. solution

* 'Roy. Soc. Proc.,' vol. 71, 1903, p. 77 (with Sydney Rowland).
† 'Lancet,' 1906, vol. 2, p. 494.
‡ 'Centralbl. f. Bakt.,' Abt. I (Originale), vol. 43, 1907, p. 143.
§ 'Brit. Med. Journ., 1906, vol. 2, p. 776.
‖ 'Roy. Soc. Proc.,' vol. 71, 1903, p. 351, and vol. 77, 1906, p. 548.
¶ 'Lancet,' 1906, vol. 2, p. 494.
** 'Lancet,' 1906, vol. 1, p. 373.
†† 'Centralbl. für Bakteriol.,' Abt. I, vol. 30, 1901, p. 368.
‡‡ See 'The Cell as the Unit of Life' (Churchill, 1908), p. 274.

(calculated on the original weight of the moist bacterial paste), and filtered through a sterile Berkefeld filter. One to three cubic centimetres of the filtered solution are then dried *in vacuo* over sulphuric acid and weighed, so as to ascertain the weight of material contained in the endotoxin solution. This weight is regarded as the weight of endotoxin; actually the endotoxin is slightly less than is represented by this weight, in consequence of the presence of traces of salts. The amount of endotoxin having been thus ascertained, sufficient sterile 0·8-per-cent. sodium chloride solution is added to the filtered solution of endotoxin so as to form a 1-per-mille solution. All the operations are performed aseptically, in order to obtain a sterile preparation.

The rabbits were all large healthy animals, and blood was obtained in Wright's capsules from an ear vein. In all instances, the blood used as the control was taken at the same time as the samples from the inoculated animals, and the specimens for counting the number of bacteria ingested by the polymorphonuclear leucocytes were made in the usual manner within two to three hours after bleeding the animals. The leucocytes employed were *human* leucocytes, as rabbit's leucocytes were found to be less satisfactory for making the stained films, and the counts were made on 50 cells. All the inoculations of endotoxin, tuberculin, and vaccine were made subcutaneously in the back.

Results obtained.

A. *Typhoid endotoxin.*—The determination of the effect of injections of typhoid endotoxin on the opsonising action of rabbit's serum is complicated by the fact that agglutinins and bacteriolytic substances are formed which cause agglutination and solution of the organisms (typhoid bacilli) in the mixtures of serum, leucocytes, and organisms employed for preparing the films with which the counts are made. The results, therefore, in this case must be regarded as approximate only. The endotoxin was prepared from an avirulent strain of the typhoid bacillus. Three rabbits were taken, one being kept as a control, the two others each receiving a dose of 0·1 milligramme of endotoxin.

In addition to determining the opsonising action of the undiluted serum, the effect of dilution was also studied, for Klien* has shown that dilution up to a certain point *increases* the opsonising action of human typhoid serum.

The following results were obtained:—

* 'Bull. of the Johns Hopkins Hospital,' vol. 18, Nos. 195 and 196, 1907, p. 245.

Table I.—Number of Typhoid Bacilli ingested by 50 Polymorphonuclear Leucocytes.

Period.	Dilution of serum.	Control (opsonic index = 1·0).	Rabbit I.	Opsonic index.	Rabbit II.	Opsonic index.
Control before inoculation	undiluted	96	82	0·85	88	0·9
	1 in 5	60	54	0·9	54	0·9
	1 10	38	42	1·1	36	1·0
24 hours after inoculation	undiluted	112	20	0·17	28	0·25
	1 in 5	86	12	0·13	14	0·16
	1 10	54	0	—	0	
48 hours after inoculation	undiluted	122	166	1·4	156	1·3
	1 in 5	74	398	5·4	336	4·5
	1 10	43	154	3·6	166	3·8
3 days after inoculation	undiluted	112	357	3·2	369	3·3
	1 in 5	70	204	3·0	254	3·6
	1 10	35	114	3·3	116	3·3
	1 20	—	70	—	84	
5 days after inoculation	undiluted	196	392	2·0	327	1·7
	1 in 5	48	548	11·4	496	10·3
	1 10	33	515	15·6	448	13·6
	1 20	14	325	23·0	357	25·5
	1 50	—	101	—	48	
6 days after inoculation	undiluted	135	103	0·8	131	1·0
	1 in 5	64	270	4·2	160	2·5
	1 10	33	124	3·7	135	4·0
	1 20	—	98	—	75	
	1 50	—	83	—	80	
	1 100	—	66	—	29	
7 days after inoculation	undiluted	114	155	1·4	125	1·1
	1 in 5	49	110	2·3	71	1·4
	1 10	28	71	2·4	42	1·5
	1 20	—	50	—	60	
	1 50	—	35	—	29	
	1 100	—	29	—	22	
8 days after inoculation	undiluted	121	202	1·7	121	1·0
	1 in 5	110	240	2·2	145	1·3
	1 10	51	168	3·3	198	3·9
	1 20	—	164	—	126	
	1 50	—	98	—	45	
12 days after inoculation	undiluted	118	—		112	0·95
	1 in 5	60	—		112	1·9
	1 10	23	—		180	7·8
	1 20	—	—		164	
	1 50	—	—		98	
	1 100	—	—		28	

It is not suggested either in Table I or in Table II that the index is correct to the second decimal. The figure in the second decimal place is given only to indicate the *trend* of the index.

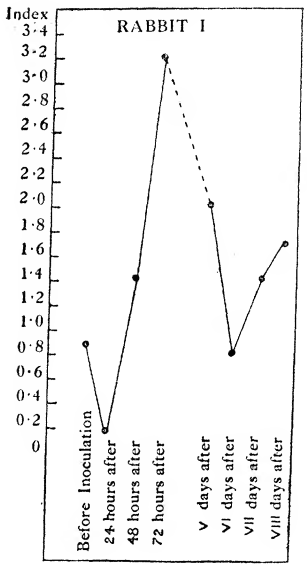

CHART I.—Opsonic Index after Inoculation with 0·1 mgrm. Typhoid Endotoxin.

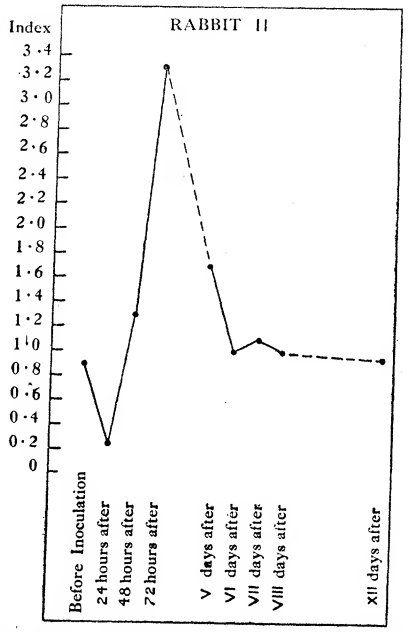

CHART II.—Opsonic Index after Inoculation with 0·1 mgrm. Typhoid Endotoxin.

From the foregoing table it is evident that an injection of 0·1 milligramme of typhoid endotoxin produces, 24 hours after inoculation, a considerable decrease in the opsonising action of the serum, that is a marked "negative phase" (Wright), followed by a considerable rise in the opsonising action of the serum which persists for some days. The opsonic index yielded by the undiluted serum is given in graphic form in Charts I and II. The dilution of the normal serum produces a decrease in its opsonising action, whereas a dilution of the serum of the inoculated rabbits produces an apparent increase in the opsonising action.

B. *Staphylococcus endotoxin.*—Three sets of experiments were carried out with the endotoxin of the *M. pyogenes*, var. *aureus* (*Staphylococcus pyogenes aureus*), viz., a comparison of the effects of the endotoxin derived from (*a*) an ordinary old laboratory strain of the organism, (*b*) a recently isolated strain, and (*c*) the effect of a vaccine prepared from the strain used for *a*, on the opsonising action of the serum of normal rabbits. Equivalent quantities (0·1 milligramme solid matter) both of vaccine and of endotoxin were given, and the endotoxin solution was prepared with 0·1-per-cent. sodium hydrate solution. The opsonising action of each serum, some time after inoculation, was also tested with both strains of organisms. The results obtained are given graphically in Chart III of the opsonic indexes.

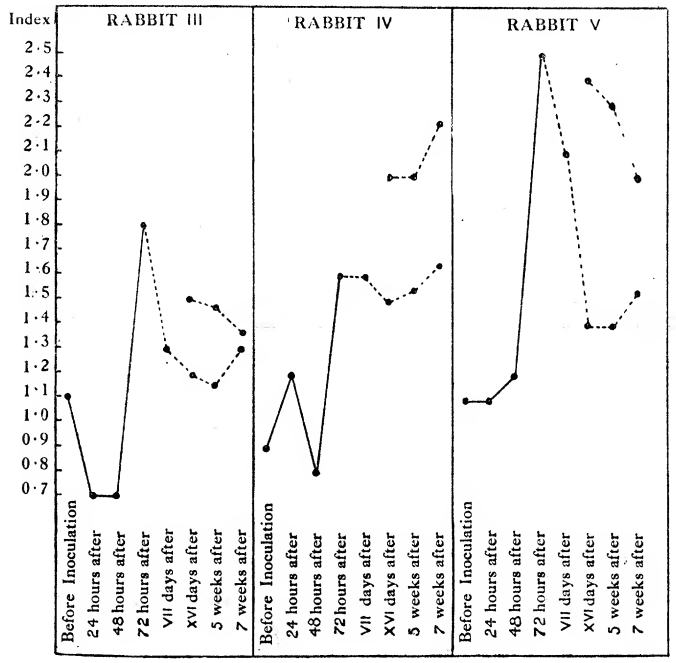

CHART III.—Staphylococcus Vaccine and Endotoxins.

Rabbit III received 1 c.c. (= 1000×10^6 = 0·1 milligramme) Staphylococcus Vaccine.
Rabbit IV received 0·1 milligramme Staphylococcus Endotoxin, *old* strain.
Rabbit V received 0·1 milligramme Staphylococcus Endotoxin, *new* strain.

Main trace = index determined with old strain of Staphylococcus.
Small upper trace = index determined with new strain of Staphylococcus.

It will be seen from these experiments that the endotoxin prepared from the old laboratory strain (Rabbit IV) gave nearly as marked a rise in the opsonic index as the vaccine (Rabbit III), but that the former seems to induce less negative phase than the latter, and its effect is more persistent. The endotoxin prepared from the recently isolated strain (Rabbit V) induced a rise in the opsonic index much more marked than that induced by either the vaccine or the endotoxin prepared from the old laboratory strain. The sera, some time after inoculation (two to seven weeks), tested against the recently isolated strain, gave an opsonic index slightly higher in the case of the vaccine (Rabbit III) and much higher in the case of the endotoxins (Rabbits IV and V) than that obtained when tested against the old laboratory strain.

The effects of different amounts (0·1, 0·01, 0·001 milligramme) of another freshly prepared staphylococcus endotoxin solution were also tested, and the results are given graphically in Chart IV of the opsonic indexes, and are

there compared with the injection of an ordinary dose of staphylococcus vaccine (1·0 c.c. = 1000×10^6 cocci = 0·1 milligramme).

From this chart (IV) it will be seen that a marked rise in the opsonic index results from the injection of staphylococcus endotoxin, and that the rise corresponds with the dose of endotoxin given. Even the smallest dose of endotoxin (0·001 milligramme, Rabbit IX), produced a considerable and lasting rise in the index, a rise more marked than in the case of the vaccine (Rabbit VI).

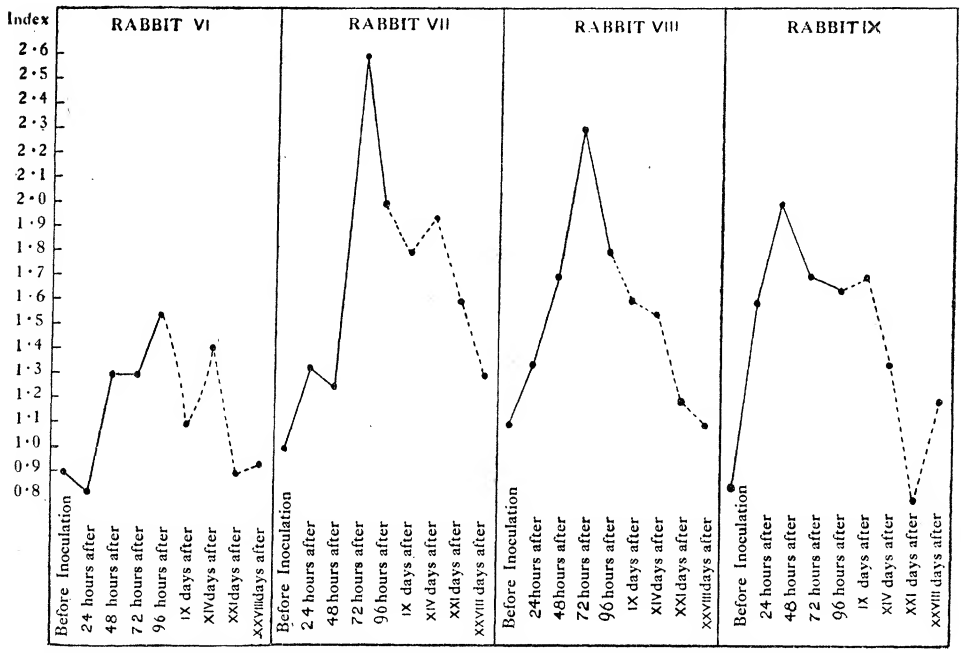

CHART IV.—Staphylococcus Vaccine and varying doses of Staphylococcus Endotoxin.

Rabbit VI received 1·0 c.c. (1000×10^6 cocci = 0·1 milligramme) Staphylococcus vaccine.
Rabbit VII received 0·1 milligramme Staphylococcus endotoxin.
Rabbit VIII received 0·01 milligramme Staphylococcus endotoxin.
Rabbit IX received 0·001 milligramme Staphylococcus endotoxin.

A few experiments on the effect of dilution on the opsonic action of a vaccine serum and of an endotoxin serum were also made, and the results obtained are given in Table II.

From Table II it will again be seen that the endotoxin produces a greater rise in the opsonic index than the vaccine does. In this case dilution does not affect the index in the same way as dilution of typhoid serum; on the whole the index remains much the same in the undiluted and the diluted serum, though with dilutions of 1 in 5 and 1 in 10 more cocci are ingested

by the leucocytes than when the serum is undiluted, and this applies to the serum both of the inoculated, and of the uninoculated, animals.

Table II.—Effects of Dilution on Staphylococcus Vaccine and Endotoxin Sera. Number of Cocci ingested by 50 Polymorphonuclear Leucocytes.

Period.	Dilution of serum.	Control (opsonic index = 1).	Vaccine serum (1 c.c. vaccine).	Opsonic index.	Endotoxin serum (0·1 mgrm.).	Opsonic index.
Control before inoculation	undiluted	210	235	1·1	199	0·95
	1 in 5	335	356	1·06	315	0·94
	1 10	432	410	0·95	397	0·92
24 hours after inoculation	undiluted	233	183	0·8	400	1·3
	1 in 5	286	256	0·9	241	0·85
	1 10	295	190	0·64	214	0·7
	1 20	108	121	1·1	144	1·3
48 hours after inoculation	undiluted	85	131	1·5	326	3·8
	1 in 10	100	169	1·7	358	3·6
72 hours after inoculation	undiluted	58	92	1·6	156	2·7
	1 in 10	80	133	1·66	131	1·6
6 days after inoculation	undiluted	125	186	1·5	306	2·4
	1 in 10	185	211	1·14	365	2·0
12 days after inoculation	undiluted	102	131	1·3	177	1·75

(See Note at end of Table I.)

C. *Bacillus tuberculosis.*—Two preparations of tubercle endotoxin were used, one prepared from untreated tubercle bacilli, the other prepared from tubercle bacilli previously extracted with ether. The effect produced by the endotoxins was compared with that produced by a small dose of German Tuberculin R. The results obtained are charted graphically in Chart V of the opsonic indexes.

From Chart V it will be seen that the German Tuberculin R (dose 0·002 milligramme) produced little effect (Rabbit X). A corresponding dose of tubercle endotoxin (prepared with *unextracted* bacilli), on the other hand, induced a marked and prolonged rise in the opsonic index, which was preceded by a slight negative phase (Rabbit XI). A relatively huge dose of the same endotoxin (1 milligramme) produced a decided negative phase followed by a rise in the opsonic index of approximately the same amount as that produced by the smaller dose (Rabbit XII). A similar dose (1 milligramme) of the endotoxin prepared with ether-extracted tubercle bacilli

produced an alteration (negative phase and subsequent rise) less marked than with the endotoxin prepared from the unextracted bacilli (Rabbit XIII).

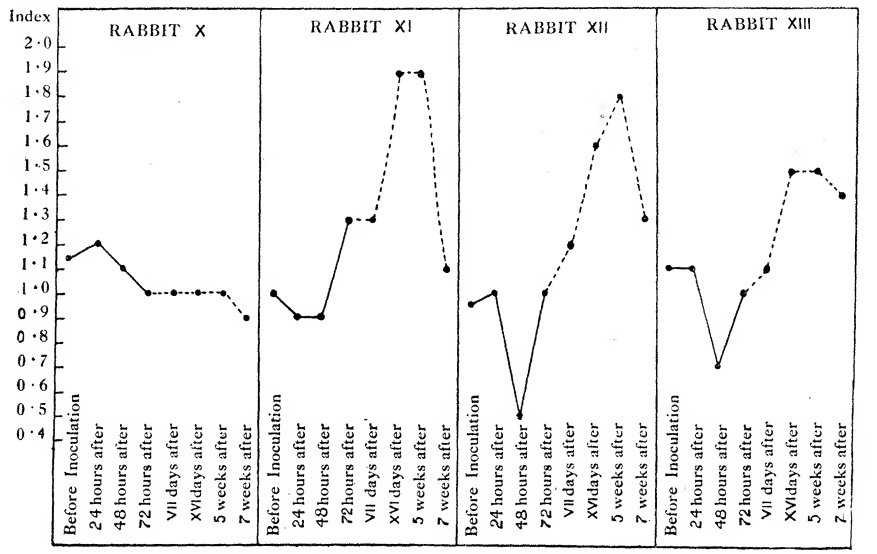

CHART V.—Tuberculin and Tubercle Endotoxin.

Rabbit X received 0·002 milligramme Tuberculin R.
Rabbit XI received 0·002 milligramme tubercle endotoxin.
Rabbit XII received 1·0 milligramme tubercle endotoxin.
Rabbit XIII received 1·0 milligramme ether-extracted tubercle endotoxin.

D. *Effect of keeping on the Activity of the Endotoxin Solutions.*—In view of the possible use of endotoxin solutions for vaccine treatment, it was thought desirable to make tests on their activity after they had been kept for a period. The tubercle endotoxin was prepared on March 9, 1908, and the staphylococcus endotoxin was prepared on March 10, 1908. They were the same solutions as those employed in the experiments detailed in Sections B and C above, were kept in an ice-safe and were injected on May 1, 1908, into fresh rabbits, *i.e.* approximately seven weeks after preparation. The results are given graphically in Charts VI and VII of the opsonic indexes.

In the case of the tubercle endotoxin, doses similar to those given in the experiments in Section C were administered. It will be seen from Chart VI of the opsonic indexes that the endotoxin solutions were quite as active as previously. Rabbit XV, receiving the large dose of endotoxin prepared from unextracted bacilli, became unwell on the twelfth day, and died on the thirty-sixth day, after injection.

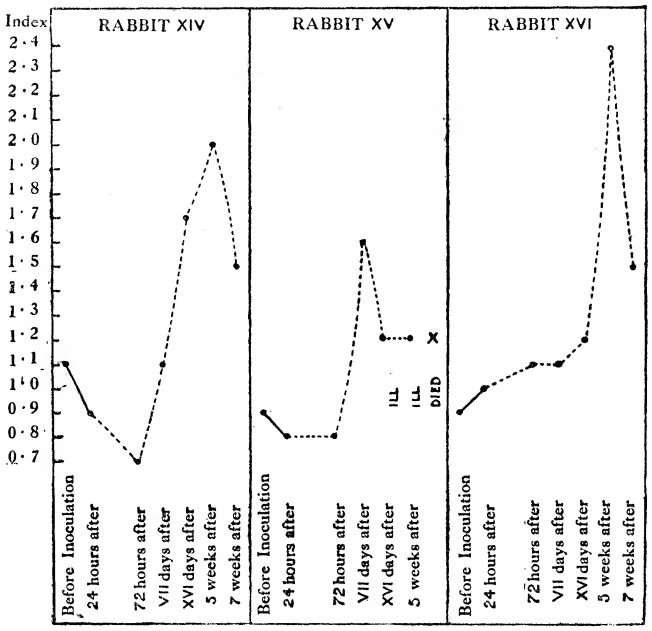

CHART VI.—*Old* Tubercle Endotoxin Solutions (7 weeks old).

Rabbit XIV received 0·002 milligramme tubercle endotoxin.
Rabbit XV received 1·0 milligramme tubercle endotoxin.
Rabbit XVI received 1·0 milligramme ether-extracted tubercle endotoxin.

In the case of the staphylococcus endotoxin, in view of possible deterioration on account of keeping, 10 times the dose (1 milligramme) previously given was injected. It will be seen from Chart VII of the opsonic indexes that a marked effect on the opsonic index was induced, greater, as before, in the case of the endotoxin prepared from the recently isolated strain (Rabbit XVIII).

Another experiment with the latter endotoxin (prepared on March 9, 1908) was performed on November 3, 1908, approximately eight months after preparation, the dose being 0·1 milligramme. Again it will be seen from Chart VIII of the opsonic indexes that a marked effect was produced (Rabbit XIX). The staphylococcus endotoxin employed in the experiment detailed in Section E below was the same preparation and was then 10 months old, and from Chart IX it will be seen that it was still very active.

These experiments indicate that the endotoxin solutions deteriorate but slowly, and retain a considerable proportion of their activity for at least three to six months.

E. *Production of "Negative Phase" by Injection of Endotoxin.*—It was

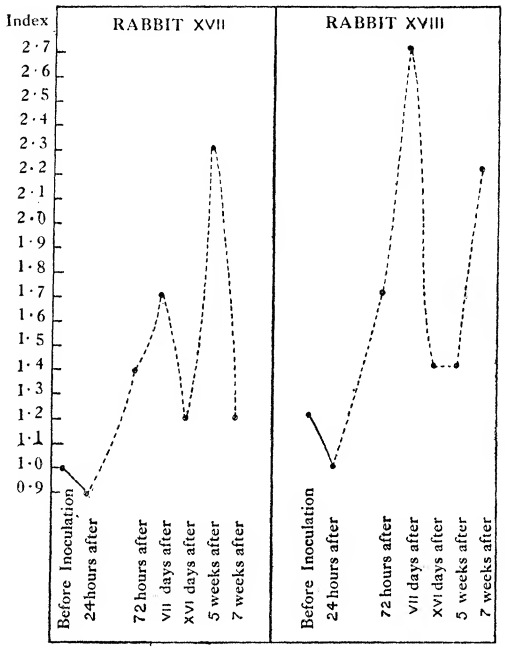

CHART VII.—*Old* Staphylococcus Endotoxin Solutions (7 weeks old).

Rabbit XVII received 1·0 milligramme Staphylococcus endotoxin, *old* strain.
Rabbit XVIII received 1·0 milligramme Staphylococcus endotoxin, *new* strain.

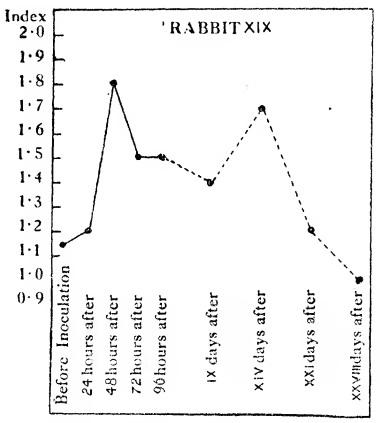

CHART VIII.—Old Staphylococcus Endotoxin Solution (8 months old). (Prepared from *new* strain of Staphylococcus.) Rabbit XIX received 0·1 milligramme endotoxin.

considered desirable to attempt to ascertain whether the endotoxin produces a negative phase comparable to that produced by a vaccine. For this purpose relatively large doses of staphylococcus vaccine ($15{,}000 \times 10^6$ cocci = 1·5 c.c. vaccine), and of fresh staphylococcus endotoxin (1 milli-

gramme) were injected, and the opsonic index was determined 15 hours, 20 hours, 24 hours, 48 hours, and 72 hours, and five days and seven days after inoculation. The results are given graphically in Chart IX of the opsonic indexes.

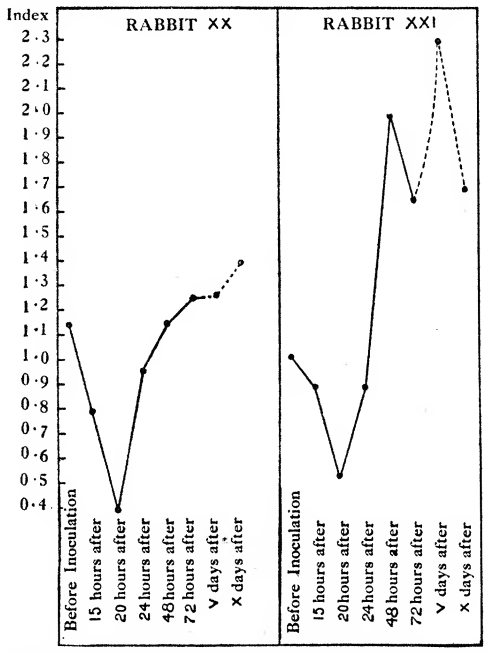

CHART IX.—To ascertain extent of "Negative Phase" with large doses of Staphylococcus Vaccine and Endotoxin.

Rabbit XX received 1·5 c.c. ($= 15 \times 10^6$ cocci = 0·15 milligramme) vaccine.
Rabbit XXI received 1·0 milligramme endotoxin.

From these experiments (Chart IX) it would appear that the vaccine (Rabbit XX) produces a decidedly greater negative phase at the twentieth hour after injection than the endotoxin does (Rabbit XXI), although, weight for weight, six and a half times as much active material was administered in the case of the endotoxin than in that of the vaccine. The results of this experiment (and also of those detailed in Sections B, C, and D) suggest that the endotoxin induces decidedly less negative phase than a vaccine.

I have to express my best thanks to Mr. Wellcome for the facilities he has afforded me at the Wellcome Physiological Research Laboratories for carrying out the greater part of this work, and to Mr. E. Thompson, Laboratory Assistant, on whom much of the labour of making the counts of the opsonic determinations has fallen.

(Part of the expense incurred in this work has been defrayed by a grant from the British Medical Association.)